ABSTRACT

We determined whether concurrent blockage of vascular endothelial growth factor (VEGF) receptor and epidermal growth factor (EGF) receptor signaling by two novel tyrosine kinase inhibitors, PTK 787 and PKI 166, respectively, can inhibit angiogenesis and, hence, the growth and metastasis of human pancreatic carcinoma in nude mice. Highly metastatic human pancreatic carcinoma L3.6pl cells were injected into the pancreas of nude mice. Seven days later, groups of mice began receiving oral doses of PTK 787 and PKI 166 three times weekly. Some groups of mice also received i.p. injections of gemcitabine twice a week. The mice were necropsied when the control mice became moribund. Treatment with PTK 787 and PKI 166, with gemcitabine alone, or with the combination of PTK 787, PKI 166, and gemcitabine produced 69, 50, and 97% reduction in the volume of pancreatic tumors, respectively. Administration of protein tyrosine kinase inhibitors and gemcitabine also significantly decreased the incidence of lymph node and liver metastasis. The therapeutic efficacy directly correlated with a decrease in circulating proangiogenic molecules (VEGF, interleukin-8), a decrease in microvessel density, a decrease in proliferating cell nuclear antigen staining, and an increase in apoptosis of tumor cells and endothelial cells. Therapies produced by combining gemcitabine with either PKI 166 or PTK 787 were similar to those produced by combining gemcitabine with both PKI 166 and PTK 787. These results suggest that blockade of either epidermal growth factor receptor or VEGF receptor signaling combined with chemotherapy provides an effective approach to the therapy of pancreatic cancer.

INTRODUCTION

Cancer of the exocrine pancreas is characterized by extensive local invasion and early lymphatic and hematogenous metastasis (1, 2). At the time of diagnosis, >80% of patients present with either locally advanced or metastatic disease (3). The inability to detect pancreatic cancer at an early stage, the aggressiveness of the disease, and the lack of effective systemic therapies are responsible for rapid death from this disease: only 1–4% of all patients with adenocarcinoma of the pancreas will survive 5 years after diagnosis (4, 5). For patients with advanced pancreatic cancer, even the recent introduction of the deoxycytidine analogue gemcitabine does not extend median survival beyond 6 months (6). Clearly, more effective therapy of human pancreatic carcinoma is urgently needed.

One mechanism that has received growing experimental and therapeutic interest is angiogenesis, which is based on the discovery that the progressive growth of cancer depends on induction of neovascularization (7–11). The extent of angiogenesis depends, in turn, on the balance between proangiogenic and antiangiogenic factors released by tumor and host cells (7–10). Human pancreatic cancer cells secrete the proangiogenic molecules VEGF, IL-8, and bFGF (12, 13). The expression of VEGF, at present regarded as the major proangiogenic factor for most types of human cancer (14), is strongly induced by EGF and tumor growth factor-α (15, 16), and overexpression of EGF, tumor growth factor-α, and EGF-R by human pancreatic tumors has been shown to correlate with rapid progression of the disease (17). Because VEGF and its receptors (Flk-1/KDR and Flt-1) and EGF and its receptor (EGF-R) play key roles in neoplastic angiogenesis (17–20), inhibition of these factors or their receptors has been a target for therapy.

Present antiangiogenic strategies aimed at inhibiting VEGF and EGF activity include VEGF-R (21–25) and EGF-R (26, 27) tyrosine kinase inhibitors. PTK 787/ZK22584 (PTK 787; 1-[4-chloroaminol]-4[pyridyl]-methyl phthalazine dihydrochloride), the inhibitor of VEGF-R tyrosine kinase, has been shown to be active in submicromolar concentrations (24, 28, 29); at higher concentrations, it inhibits other class III kinases, such as c-kit and fms, but not kinases from other receptor families, e.g., EGF-R, c-met, and Tek. PKI 166 [4-(R)-phenethylamino-6-(hydroxyl)phenyl-7H-pyrido[2,3-d]-pyrimidine], an inhibitor of the pyrrolopyrimidine class, has been shown to inhibit the intracellular domain of the EGF-R kinases with an IC50 of 0.7 nm and is less selective against other tyrosine kinases, such as c-Src and KDR (30).

Recent work from our laboratory demonstrated that daily oral administrations of PTK 787 (25) or oral administration of PKI 166 three times a week (26, 27), each combined with gemcitabine, could inhibit the growth and metastasis of human pancreatic carcinoma implanted orthotopically in nude mice. IHC analysis of pancreatic tumors from mice treated with PKI 166 revealed that the decrease in activated EGF-R was accompanied by a decrease in the expression of the proangiogenic molecules VEGF and IL-8. We now report that oral administrations of PTK 787 and PKI 166 three times a week combined with i.p. injections of gemcitabine twice a week significantly retard the growth of human pancreatic carcinoma implanted in the pancreas of nude mice. The mechanism of this combination is the induction of apoptosis in both tumor cells and tumor-associated endothelial cells.

MATERIALS AND METHODS

Pancreatic Cancer Cell Lines and Culture Conditions. The highly metastatic human pancreatic cancer cell line L3.6pl (12) was maintained in DMEM supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, t-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO2 and 95% air. The cultures were free of Mycoplasma and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theliers’ encephalitis virus, Sendai virus, minute virus, mouse adenoavirus, mouse hepatitis virus, lympho-
cytic choriomeningitis virus, extromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 10 weeks after recovery from frozen stocks.

**Reagents.** PTK 787, a novel VEGF-R tyrosine kinase inhibitor, was discovered and synthesized in the Department of Oncology Research, Novartis Pharma (Basel, Switzerland) and was profiled in collaboration with the Institute of Molecular Medicine, Tumor Biology Center (Freiburg, Germany), as well as the Oncology Research Laboratories of Schering AG (Berlin, Germany; Ref. 28). The studies described in this report were performed with a dihydrochloride salt. For *in vivo* administration, PTK 787 was dissolved in distilled water. PKI 166, a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG. For *in vivo* administration, PKI 166 was dissolved in DMSO-0.5% Tween 80 and then diluted 1:20 in water (30). All purchased reagents (e.g., VEGF/RhVEGF or monoclonal antibodies) were obtained from commercial suppliers. PKI 166, a novel EGF-R tyrosine kinase inhibitor, was synthesized and covered in the Department of Oncology Research, Novartis Pharma (Basel, Switzerland) and was profiled in collaboration with the Institute of Molecular Medicine, Tumor Biology Center (Freiburg, Germany), as well as the Oncology Research Laboratories of Schering AG (Berlin, Germany; Ref. 28). The studies described in this report were performed with a dihydrochloride salt. For *in vivo* administration, PTK 787 was dissolved in distilled water. PKI 166, a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG. For *in vivo* administration, PKI 166 was dissolved in DMSO-0.5% Tween 80 and then diluted 1:20 in water (30). All purchased reagents (e.g., VEGF/RhVEGF or monoclonal antibodies) were obtained from commercial suppliers. PKI 166, a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG. For *in vivo* administration, PKI 166 was dissolved in DMSO-0.5% Tween 80 and then diluted 1:20 in water (30). All purchased reagents (e.g., VEGF/RhVEGF or monoclonal antibodies) were obtained from commercial suppliers.

**Animals and Orthotopic Implantation of Tumor Cells.** Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions. The facilities were approved by the American Association for Accreditation of Laboratory Animal Care and meet all present regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8–12 weeks of age.

To produce tumors, L3.6pl cells (12) were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only single-cell suspensions of >90% viability were used for the injections. Injection of cells into the pancreas was performed as described previously (12). Briefly, male nude mice were anesthetized with methoxyflurane. A small left abdominal flank incision was made, and the spleen was exteriorized. Tumor cells (1 × 10^7–10^8) were injected subcapsularly in a region of the pancreas just beneath the spleen. We used a 30-gauge needle, a 1-ml disposable syringe, and a calibrated, push-button-controlled dispensing device to inject the tumor cell suspension (Hamilton Syringe Co., Reno, NV). A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without i.p. leakage. To prevent such leakage, a cotton swab was held for 1 min over the site of injection. One layer of the abdominal wound was closed with wound clips (Auto-clip; Clay Adams, Parsippany, NJ). The animals tolerated the surgical procedure well, and no anesthesia-related deaths occurred (12).

**Therapy for Established Human Pancreatic Carcinoma Tumors Growing in the Pancreas of Athymic Nude Mice.** Seven days after the implantation of tumor cells into the pancreas, five mice were killed, and the presence of tumor lesions was determined. At that time, the median tumor volume was 18 mm³. Histological examination confirmed that the lesions were actively growing adenocarcinoma. The mice were randomized into four groups (n = 10) as follows: (a) oral administration of vehicle solutions for PTK 787 (water) and PKI 166 (DMSO-0.5% Tween 80 diluted 1:20 in water) and i.p. injection of HBSS (control group); (b) i.p. injections of 125 mg/kg gemcitabine (26) twice a week (Tuesday and Thursday); (c) oral administrations of 50 mg/kg PTK 787 and 50 mg/kg PKI 166 three times per week (Monday, Wednesday, and Friday); and (d) oral administration of 50 mg/kg PTK 787 and 50 mg/kg PKI 166 three times per week and i.p. injections of 125 mg/kg gemcitabine (26) twice a week. PTK 787 and PKI 166 were administered together.

**Necropsy Procedures and Histological Studies.** Mice were killed by CO2 and weighed. Primary tumors in the pancreas were excised and weighed. For IHC and H&E staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, another part was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Visible liver metastases were counted with the aid of a dissecting microscope and processed for H&E staining. All macroscopically evident regional (celiac and para-aortal) lymph nodes were harvested, and the presence of metastatic disease in these lymph nodes was confirmed by histological examination.

**IHC Determination of VEGF, IL-8, PCNA, and CD31/PECAM-1.** Paraffin-embedded tissues were used for identification of VEGF, IL-8, and PCNA. Sections (4–6 μm thick) were mounted on positively charged Super-fridge slides (Fisher Scientific Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene and then treated with a graded series of alcohol [100, 95, and 80% ethanol (v/v) in doubly distilled H2O] and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA were microwaved 5 min for “antigen retrieval” (31). All other paraffin-embedded tissues were treated with pepsin (Biomeda) for 15 min at 37°C and washed with PBS (32). Frozen tissues used for identification of CD31/PECAM-1 were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), 1% acetic acid-chloroform (v/v; 5 min), and acetone (5 min) and washed with PBS. IHC procedures were performed as described previously (33). A positive reaction was visualized by incubating the slides with stable 3,3-diaminobenzidine (Research Genetics, Huntsville, AL); 3-amino-9-ethylcarbazole (Biogenex Laboratories, San Ramon, CA); and Gill’s hematoxylin (Sigma Chemical Co., St. Louis, MO). Prolong solution was purchased from Moleculal Probes (Eugene, OR). Pepsin was purchased from Biomeda (Foster City, CA).

**Immunofluorescence Microscopy.** The equilibration buffer was drained, and reaction buffer containing equilibration buffer containing goat antimouse IgG F(ab')2 fragment (1:10 dilution in PBS) for 4–6 h before incubation with the primary antibody. Control sections exposed to secondary antibody alone showed no specific staining.

**Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells).** Frozen tissues were sectioned (8–10 μm), mounted on positively charged slides, air-dried for 30 min, and fixed in cold acetone for 5 min, in 1:1 acetic acid-chloroform (v/v; 5 min), and in acetone for 5 min. Samples were washed three times with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and then incubated with the appropriate dilution (1:400) of secondary goat antirat antibody conjugated to Texas Red for 1 h at 4°C. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics). No counterstaining was used for EGFR, EGF-R, or activated EGF-R. Sections analyzed for activated EGF-R were pretreated with goat antimouse IgG F(ab')2 fragment (1:10 dilution in PBS) for 4–6 h before incubation with the primary antibody. Control sections exposed to secondary antibody alone showed no specific staining.

**Necropsy Procedures and Histological Studies.** Mice were killed by CO2 and weighed. Primary tumors in the pancreas were excised and weighed. For IHC and H&E staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, another part was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Visible liver metastases were counted with the aid of a dissecting microscope and processed for H&E staining. All macroscopically evident regional (celiac and para-aortal) lymph nodes were harvested, and the presence of metastatic disease in these lymph nodes was confirmed by histological examination.
filters mounted in a filter wheel (Ludi Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured with a Sony 3-chip camera (Sony Corporation of America, Montvale, NJ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas Image Analysis software (Bioscan, Edmond, WA) installed on a Compaq computer with Pentium chip, a frame grabber, an optical disc storage system, and a Sony MAVigraph UP-D7000 digital color printer (Tokyo, Japan). Images were further processed using Adobe PhotoShop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to total number of endothelial cells in 5–10 random 0.011-mm² fields at ×400 magnification. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random 0.159-mm² fields at ×100 magnification.

Quantification of MVD, PCNA, and Absorbance. For quantification of MVD, 10 random 0.159-mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified according to the method described previously (34, 35). For quantification of the IHC reaction intensity, the absorbance of 100 VEGF- and IL-8-positive cells from each of the differently treated tumor tissues was measured in 10 random 0.039-mm² fields at ×100 magnification using the Optimas Image Analysis software (12, 13, 36). The samples were not counterstained, so that the absorbance was attributable solely to the product of the IHC reaction. VEGF and IL-8 cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and was expressed as a ratio of tumor cell expression to normal pancreatic gland expression multiplied by 100 (34, 35). The number of PCNA-positive cells was quantified in 10 random 0.159-mm² fields at ×100 magnification.

Assay of VEGF, bFGF, and IL-8 Plasma Levels. Blood was collected 6 h after the last treatment. Levels of VEGF, bFGF, and IL-8 protein in the plasma were determined by ELISA kits according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Statistical Analysis. The volume of pancreatic tumors, expression of VEGF and IL-8, quantification of PCNA, TUNEL, CD31, and percentage of apoptotic endothelial cells were compared by the unpaired Student’s t test. Incidence of liver metastasis between groups was compared using the Fisher’s exact test.

RESULTS

Inhibition of Growth and Metastasis of Human L3.6pl Pancreatic Cancer in Nude Mice. We injected 1 × 10⁶ viable L3.6pl cells into the pancreas of athymic nude mice (12). Seven days later, the mice were randomized into four treatment groups of 10 mice each. The first group (control) received oral vehicle solution for PTK 787, and the second group received i.p. injection of gemcitabine (125 mg/kg). The third group received oral doses of 50 mg/kg PTK 787 and 50 mg/kg PKI 166 three times a week, and the fourth group received oral doses of 50 mg/kg PTK 787 and 50 mg/kg PKI 166 three times a week and i.p. injections of 125 mg/kg gemcitabine twice a week.

All mice were killed on day 35 because the control mice were moribund at that time.

Previous results from our laboratory demonstrated that oral administration of PTK 787 to nude mice with L3.6pl cells implanted in the pancreas significantly decreased the median tumor volume, from 668 mm³ to 227 mm³ (P < 0.01; Ref. 25). Oral administration of PKI 166 significantly decreased the median volume of pancreatic tumors, from 574 mm³ to 174 mm³ (P < 0.001; Ref. 27). In the present study, we determined whether concurrent oral administration of PTK 787 and PKI 166 could produce superior effects. In one representative experiment (summarized in Table 1), oral administrations of PTK 787 and PKI 166 three times a week and i.p. injections of gemcitabine twice a week significantly decreased the median pancreatic tumor volume compared with control mice (232, 380, and 758 mm³, respectively; P < 0.01). The combination of PTK 787, PKI 166, and gemcitabine produced an even greater decrease in median volume of pancreatic tumors (to 21 mm³; P < 0.001). Visible liver metastases (enumerated with the aid of a dissecting microscope) were present in 40% of control mice and 0–20% of the treatment groups (Table 1). Histologically positive regional lymph node metastases were found in 90% of control animals, in 80% of gemcitabine-treated animals, in 90% of the animals treated with PTK 787 plus PKI 166, and in only 20% of animals receiving PTK 787, PKI 166, and gemcitabine (Table 1). No metastases were detected in any other organ.

Treatment with PTK 787 and PKI 166 alone or in combination with gemcitabine was well tolerated, as shown by the essentially equivalent body weights in all groups (Table 1).

Histology and IHC Analyses. Pancreatic tumors were processed for routine histology and IHC analyses. Immunohistochemistry using specific anti-EGF and specific anti-EGF-R antibodies as well as antibodies specific against tyrosine phosphorylated (activated) EGF-R demonstrated that tumors from all treatment groups expressed similar levels of EGF and EGF-R, whereas only tumors from control mice or mice treated with gemcitabine stained positive for activated EGF-R (Fig. 1).

We next evaluated cell proliferation and apoptosis, using anti-PCNA antibodies and the TUNEL method, respectively (Fig. 2). The mean number of PCNA-positive tumor cells in pancreatic tumors of control mice was 380 ± 28. After therapy with gemcitabine or PTK 787 plus PKI 166, it was 175 ± 31 and 46 ± 5, respectively (Table 2). The lowest number of PCNA-positive cells (36 ± 5) was found in tumors of mice treated with PTK 787, PKI 166, and gemcitabine (P < 0.001 versus control).

The mean number of TUNEL-positive cells inversely correlated with PCNA positivity. In control tumors, the mean was 12 ± 6, in gemcitabine-treated tumors, it was 47 ± 13, and in PTK 787 plus PKI 166-treated tumors, it was 100 ± 19. In tumors treated with all three agents, it was 127 ± 17 (gemcitabine versus control, P < 0.01; all others versus control, P < 0.001). No significant differences in the number of TUNEL-positive tumor cells were

| Table 1 | Therapies for human pancreatic carcinoma growing in the pancreas of nude mice |
|---------|-----------------|---------------------|-----------------|
| Pancreatic tumors | Metastases (n) | Body weight (g) |
| Tumor volume (mm³) | Incidence* (n) | Median | Range | Liver* | Lymph node* | Median | Range |
| Saline control | 10/10 | 758 | 441–2688 | 4/10 | 9/10 | 21 | 18–28 |
| Gemcitabine | 9/10 | 380* | 144–968 | 2/10 | 8/10 | 23 | 22–28 |
| PTK 166 | 9/10 | 232* | 25–416 | 1/10 | 9/10 | 23 | 22–30 |
| PKI 166 + PTK 787 | 9/10 | 21* | 10–100 | 0/10 | 2/10 | 22 | 22–27 |
| PKI 166 + PTK 787 + Gemcitabine | 9/10 | 21* | 10–100 | 0/10 | 2/10 | 22 | 22–27 |

* L3.6pl human pancreatic cancer cells (1 × 10⁶) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with i.p. injections of gemcitabine (125 mg/kg) alone (twice a week), oral feedings of PKI 166 (50 mg/kg) and PTK 787 (50 mg/kg; three times a week), gemcitabine in combination with PKI 166 and PTK 787, or saline (control). All mice were killed on day 35. * Number of positive mice/number of mice injected.

** Compared with controls: * P < 0.01; ** P < 0.001.
found among the therapy groups, i.e., tumors treated with gemcitabine or PTK 787 and PKI 166 or the combination of PTK 787, PKI 166, and gemcitabine. The calculated ratio of PCNA-positive over TUNEL-positive cells was 32, 4, 0.46, and 0.28 in control mice, mice treated with gemcitabine alone, PTK 787 and PKI 166 alone, and combination therapy of all three, respectively (Fig. 2 and Table 2).

Production of VEGF and IL-8 by tumor cells was significantly reduced on day 35 in mice treated with PTK 787 plus PKI 166 or PTK 787, PKI 166, and gemcitabine compared with control mice or mice treated with gemcitabine alone, PTK 787 and PKI 166 alone, and combination therapy of all three, respectively (Fig. 2 and Table 2). Previous reports from our group have shown that oral administration of PTK 787 does not decrease the expression of VEGF, IL-8, or bFGF in treated neoplasms (24, 25), whereas oral administration of PKI 166 significantly decreases production of VEGF and IL-8 by tumor cells (26, 27). In the present study, the levels of VEGF and IL-8 in plasma were determined by ELISA (Fig. 3). Plasma from control mice contained 2000 pg/ml VEGF and 1922 pg/ml IL-8. Mice treated with PTK 787 and PKI 166 had plasma levels of 38 pg/ml VEGF ($P < 0.001$) and 1186 pg/ml IL-8. Mice treated with all three agents had negligible plasma levels of VEGF and 840 pg/ml IL-8 ($P < 0.01$). The reduced levels of VEGF and IL-8 in the tumors of mice treated with PTK 787, PKI 166, and gemcitabine correlated with a reduction of these angiogenic cytokines in the blood. Because VEGF receptors are most likely expressed on endothelial cells and not tumor cells, treatment with PTK 787 does not target tumor cells directly. Tumor cells, however, do express EGF-R, and blockade of its signaling has been shown to inhibit expression of VEGF, bFGF, and IL-8 (Fig. 2; Refs. 26, 27).

Although mice treated with gemcitabine did not show decreased expression of VEGF in the tumors (Fig. 2), we did not detect VEGF in the plasma of these mice (Fig. 3). The mechanism by which gemcitabine affected levels of circulating VEGF is unclear. MVD (measured by staining with antibodies against CD31) was directly proportional to the expression of VEGF and IL-8, i.e., we found a significant reduction in tumor MVD in tumors after treatment with PTK 787 and PKI 166 (25 ± 5) or combination therapy of all three agents (7 ± 1) compared with control tumors (95 ± 7) or gemcitabine-treated tumors (72 ± 10; control versus PTK 787 and PKI 166, $P < 0.01$; control versus PTK 787, PKI 166, and gemcitabine, $P < 0.001$; Fig. 2 and Table 2).

Finally, the CD31/TUNEL fluorescent double-labeling technique (25–27) revealed that many endothelial cells in pancreatic tumors from mice treated with PTK 787 and PKI 166 without or with gemcitabine underwent apoptosis. A significant increase in the percentage of apoptotic endothelial cells over total endothelial cells was
DISCUSSION

Dual blockade of the VEGF-R and EGF-R signaling pathways by oral administration of the VEGF-R and EGF-R tyrosine kinase inhibitors PTK 787 and PKI 166, respectively, combined with i.p injections of gemcitabine significantly inhibited growth and metastasis of human pancreatic carcinoma cells implanted into the pancreas of nude mice. Previous results from our laboratory demonstrated that oral administration of PTK 787 to nude mice implanted with L3.6pl cells into the pancreas significantly decreased the median tumor volume, from 668 mm$^3$ to 227 mm$^3$ ($P < 0.01$; Ref. 25), whereas the oral administration of PKI 166 significantly decreased the median volume of pancreatic tumors, from 574 mm$^3$ to 174 mm$^3$ ($P < 0.001$; Ref. 27). In the

found in pancreatic tumors harvested 28 days after the initiation of treatment with PTK 787 and PKI 166 (16 ± 11) or PTK 787, PKI 166 plus gemcitabine (27 ± 19) compared with control tumors or gemcitabine-treated tumors ($P < 0.01$; Fig. 2 and Table 2).

Fig. 2. IHC analysis. Tumors were harvested from control mice and mice treated with gemcitabine alone, PKI 166 plus PTK 787, or gemcitabine plus PKI 166 and PTK 787. Tissue sections were immunostained for expression of PCNA (to show proliferation); for TUNEL (FITC; to show death); for VEGF, IL-8, and CD31/PECAM-1 (to show endothelial cells and, hence, MVD); and for a double label of CD31 (endothelial cells) and TUNEL (apoptosis). Tumors from mice treated with gemcitabine, PKI 166 plus PTK 787, or a combination had a decrease in PCNA$^+$ cells and an increase in TUNEL$^+$ cells. Tumors from mice treated with all three compounds had decreased immunoreactivity for VEGF and IL-8. Treatment with all three compounds significantly decreased the number of CD31$^+$ endothelial cells. This decrease was directly correlated with induction of apoptosis. Red, CD31$^+$ endothelial cells; green, TUNEL$^+$ cells; yellow, TUNEL$^+$-CD31$^+$ cells.
present study, the oral administration of both PTK 787 and PKI 166 reduced the mean tumor volume from 668 mm$^3$ to 126 mm$^3$ ($P < 0.01$). This combination, therefore, did not produce superior therapeutic effects. Treatment of mice with gemcitabine reduced the tumor volume to 380 mm$^3$ ($P < 0.01$). In previous studies (26, 27), the combination of gemcitabine and PKI 166 reduced the mean tumor volume from 574 mm$^3$ to 27 mm$^3$ ($P < 0.001$), and gemcitabine plus PTK 787b reduced the mean tumor volume from 668 mm$^3$ to 126 mm$^3$ ($P < 0.01$). Treatment of mice with all three agents (PTK 787, PKI 166, and gemcitabine) reduced the mean tumor volume from 758 mm$^3$ to 232 mm$^3$ ($P < 0.01$). This combination, therefore, did not produce superior therapeutic effects. Treatment of mice with gemcitabine reduced the tumor volume to 380 mm$^3$ ($P < 0.01$). In previous studies (26, 27), the combination of gemcitabine and PKI 166 reduced the mean tumor volume from 574 mm$^3$ to 27 mm$^3$ ($P < 0.001$), and gemcitabine plus PTK 787b reduced the mean tumor volume from 668 mm$^3$ to 126 mm$^3$ ($P < 0.01$). Treatment of mice with all three agents (PTK 787, PKI 166, and gemcitabine) reduced the mean tumor volume from 758 mm$^3$ to 232 mm$^3$ ($P < 0.01$). This combination, therefore, did not produce superior therapeutic effects.

IHC analyses of the pancreatic cancers demonstrated significant decreases in MVD and in proliferating tumor cells with an associated increase in apoptosis of tumor cells and tumor-associated endothelial cells. In good agreement with our previous reports (26, 27), we found a significant decrease in the level of activated EGF-R in pancreatic tumors of mice treated with PKI 166. The blockade of the EGF-R signal pathway was associated with a decrease in expression of VEGF and IL-8. Moreover, double staining of endothelial cells with antibodies against CD31 and TUNEL suggested that the reduction in MVD was attributable to a significant increase of apoptosis in the endothelial cells. The induction of apoptosis was observed in the PTK 787/PKI 166 and PTK 787/PKI 166/gemcitabine-treated mice.

The interaction of VEGF and EGF with their receptors [Flk-1/KDR and Flt-1 (20, 23–25) and EGF-R (26, 27, 36), respectively] has been shown to play an important role in neoplastic angiogenesis. The coexpression of VEGF and its receptor (24, 25, 37–39) and EGF-R (26, 27, 36) with at least one of its ligands correlates with rapid progression of pancreatic cancer and decreased survival. On the basis of these prior studies, we hypothesized that dual blockade of VEGF-R and EGF-R signaling is an important antiangiogenic therapeutic modality for inhibiting the growth and preventing the metastasis of human pancreatic cancer. Our results support this hypothesis.

The two major functions of VEGF are induction of angiogenesis and vascular hyperpermeability, both of which are thought to be mediated mainly by Flk-1/KDR (40, 41). Subsequent to ligand binding, the EGF-R dimerizes to become activated through auto- and transphosphorylation (42). Activated EGF-R can also regulate apoptosis (43, 44), and inactivation of activated EGF-R inhibits EGF-induced receptor autophosphorylation, entry of cells into S-phase, cyclin E-associated kinase activity, and consequently, accumulation of cells in the $G_s$ phase of the cell cycle (45). In this study we found that treatment with PTK 787 and PKI 166 inhibited vascularization and, hence, total tumor volume and weight. This treatment was associated with a decrease in cell proliferation (PCNA$^+$) and an increase in apoptotic tumor cells (TUNEL$^+$). IHC analyses of the tumor specimens led us to conclude that treatment with PTK 787 and PKI 166 (alone or in combination with gemcitabine) also produced apoptosis in tumor-associated endothelial cells and hence a decrease in mean MVD within the tumors. The concomitant decrease in expression of VEGF and IL-8, which serve as survival factors for endothelial cells (45–54), could have also contributed to their demise.

The progressive growth of neoplasms depends on the induction of angiogenesis (10, 11). To produce new vessels, endothelial cells must migrate, degrade extracellular matrix, divide, form tubes, and survive (10, 11). Data from our laboratory (26, 27) and others (15, 16, 55) clearly show that dividing endothelial cells can express EGF-R and activated EGF-R. Because treatment with a tyrosine kinase inhibitor of EGF-R can induce apoptosis in EGF-R-positive endothelial cells (26, 27), PKI 166 can target both EGF-R-expressing tumor cells and tumor-associated endothelial cells. PTK 787 inhibits phosphorylation of VEGF-R, which is expressed on endothelial cells (14). VEGF is a survival factor for endothelial cells (10, 28, 33, 37, 40, 46).

In summary, we show that inhibition of protein tyrosine kinases of either the VEGF-R (PTK 787) or EGF-R (PKI 166) combined with gemcitabine can significantly reduce the growth and metastatic potential of highly metastatic human pancreatic cancer growing in the
pancreas of nude mice. The combination of both inhibitors does not produce additive therapeutic effects.

ACKNOWLEDGMENTS

We thank Walter Pagel for critical editorial review and Laura Longoria for expert assistance in the preparation of this manuscript.

REFERENCES


Blockade of Vascular Endothelial Growth Factor Receptor and Epidermal Growth Factor Receptor Signaling for Therapy of Metastatic Human Pancreatic Cancer

Cheryl H. Baker, Carmen C. Solorzano and Isaiah J. Fidler


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/7/1996

Cited articles
This article cites 51 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/7/1996.full.html#ref-list-1

Citing articles
This article has been cited by 48 HighWire-hosted articles. Access the articles at:
/content/62/7/1996.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.